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Salicylate Downregulates 11β-HSD1 Expression in Adipose Tissue in Obese Mice and in Humans, Mediating Insulin Sensitization


Recent trials show salicylates improve glycemic control in type 2 diabetes, but the mechanism is poorly understood. Expression of the glucocorticoid-generating enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) in adipose tissue is increased in vitro by proinflammatory cytokines and upregulated in obesity. 11β-HSD1 inhibition enhances insulin sensitivity. We hypothesized that salicylates downregulate 11β-HSD1 expression, contributing to their metabolic efficacy. We treated diet-induced obese (DIO) 11β-HSD1–deficient mice and C57Bl/6 mice with sodium salicylate for 4 weeks. Glucose tolerance was assessed in vivo. Tissue transcript levels were assessed by quantitative PCR and enzyme activity by incubation with 3H-steroid. Two weeks’ administration of salicylate was also investigated in a randomized double-blind placebo-controlled crossover study in 16 men, with measurement of liver 11β-HSD1 activity in vivo and adipose tissue 11β-HSD1 transcript levels ex vivo. In C57Bl/6 DIO mice, salicylate improved glucose tolerance and downregulated 11β-HSD1 mRNA and activity selectively in visceral adipose. DIO 11β-HSD1–deficient mice were resistant to these metabolic effects of salicylate. In men, salicylate reduced 11β-HSD1 expression in subcutaneous adipose, and in vitro salicylate treatment reduced adipocyte 11β-HSD1 expression and induced adiponectin expression only in the presence of 11β-HSD1 substrate. Reduced intra-adipose glucocorticoid re-generation by 11β-HSD1 is a novel mechanism that contributes to the metabolic efficacy of salicylates. Diabetes 61:790–796, 2012

Salicylate anti-inflammatory agents, including acetylsalicylic acid (aspirin) and salicylsalicylic acid (salsalate) (1), improve insulin sensitivity in animal models (2–5) and in healthy or obese humans (6–9) and improve glycemic control in patients with type 2 diabetes (10–12). The mechanism of insulin sensitization is uncertain and may involve blockade of inhibitor of κB kinase-β and, hence, nuclear factor-κB effects (13,3,14) and/or interference with phosphorylation and activity of CCAAT enhancer binding protein-β (a transcription factor involved in metabolic and inflammatory pathways) (15,16), with associated reductions in proinflammatory cytokines.

11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) catalyzes the regeneration of active cortisol from inert cortisone (corticosterone from 11-dehydrocorticosterone in rodents), including in liver and adipose tissue (17). In genetically modified mice, overexpression of 11β-HSD1 in adipose tissue (18) or liver (19) results in increased glucocorticoid action and insulin resistance, whereas targeted disruption of Hsd11b1 protects against insulin resistance and central obesity on high-fat feeding (20–22). Pharmacological inhibition of 11β-HSD1 lowers intracellular glucocorticoid levels and thereby enhances insulin sensitivity in rodents and humans (23–26). Circumstantial evidence suggests that salicylates may reduce 11β-HSD1 in adipose tissue. Proinflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin-1β, and interleukin-6, upregulate 11β-HSD1 in several cell types in culture, including in human preadipocytes (27–29). In human obesity, a state of systemic and intra-adipose inflammation (14), 11β-HSD1 mRNA levels and activity are decreased in liver (30,31) but increased in subcutaneous adipose tissue (32,31,33)—reviewed in (34)—and less consistently in visceral adipose tissue (35,36), explaining elevated intra-adipose cortisol regeneration (37). These observations raise the possibility that intra-adipose 11β-HSD1 expression is increased by the proinflammatory state in obesity. Indeed, in HIV lipodystrophy, a paradigm of extreme intra-adipose inflammation, there is increased intra-adipose and systemic 11β-HSD1 activity (38).

We hypothesized that salicylates reduce 11β-HSD1 expression in adipose tissue and that this contributes to their insulin-sensitizing effect. We examined the effects of salicylate on adipose tissue 11β-HSD1 in vivo in lean and obese mice and humans.

RESEARCH DESIGN AND METHODS

Chemicals were purchased from Sigma-Aldrich (Dorset, U.K.) unless otherwise stated.

Cell culture experiments. Simpson-Golabi-Behmel syndrome (SGBS) cells are a human preadipocyte cell line characterized by a high capacity for in vitro differentiation (39). Cells were cultured in Dulbecco’s modified Eagle’s medium–F12, supplemented with 10% (v/v) FCS, 50 units/mL penicillin per 50 units/mL streptomycin, biotin (33 μmol/L), and pantethonic acid (17 μmol/L) at 37°C in 5% CO2. Adipogenic differentiation was induced after reaching near confluence as described previously (39). At day 14, cells were incubated for 24 h in stripped serum medium prior to treatment with salicylic acid (10, 30, and 100 μmol/L) or vehicle (0.1% ethanol) for 24 h, and RNA was extracted for analysis of 11β-HSD1 transcript. To assess the interaction between salicylate and 11β-HSD1 in determining adiponectin (AdiQ) transcript levels, day 14 cells were incubated for 24 h in cortisol-free, stripped serum medium prior to treatment with cortisol (0.1 μmol/L), cortisone (0.1 μmol/L), or vehicle (0.1% ethanol) with or without salicylic acid alone (100 μmol/L) or vehicle (0.1% ethanol) for 24 h. RNA was extracted for analysis.

Experiments in mice. Experiments were performed under license from the U.K. Home Office. Mice were maintained under controlled conditions of light (0700–1900 h) and temperature (18–20°C) and allowed access to food and drinking water ad libitum. Adult male C57Bl/6 mice were obtained (Harlan Olac, Oxfordshire, U.K.) at age 12 weeks. C57Bl/6 Lean animals were maintained on normal chow diet (2.27% fat, 4.0% sucrose; 501151; Special Diet Services). Diet-induced obese C57Bl/6 mice (C57Bl/6 DIO) were given 10 weeks of high-fat diet (58% fat, 12% sucrose; D12331; Research Diets, New Brunswick, NJ) before...
treatment. Homozygous male 11β-HSD1-deficient (HSD1KO) mice were bred from an in-house colony on a C57Bl/6 background (>10 generations backcross) (21) and fed a high-fat diet until weight matched with C57Bl/6 DIO mice (~36 g).

Sodium salicylate (salicylate) (120 mg/kg/day) or distilled water (vehicle) was administered from 1 week after arriving (C57Bl/6 Lean), after 10 weeks of high-fat feeding (C57Bl/6 DIO), or after achieving target weight (HSD1KO-DIO) for 4 weeks to groups of n = 8 via osmotic minipumps implanted subcutaneously between the scapulae.

For glucose tolerance tests, mice were fasted for 6 h before administration of glucose (2 g/kg) by intraperitoneal injection and blood sampling by tail nick for 90 min thereafter.

Animals were killed by decapitation and tissues dissected onto dry ice and stored at −80°C until analysis.

**Experiments in humans.** A total of 16 otherwise healthy men, selected to represent a wide range of BMIs, from 20 to 50 kg/m², took part in a double-blind randomized balanced crossover study to compare salisate (Disalid, 3M Pharmaceuticals, Northridge, CA; 1 g p.o. every 8 h for 2 weeks) with placebo.

At the end of each phase, volunteers attended the clinical research facility on 2 consecutive days. On day 1, they completed a 24-h urine collection and attended at 0830 h after fasting overnight. Blood was taken for glucose, insulin, lipid profile, and plasma cortisol. Height, weight, waist, and hip circumferences; percent fat by bioimpedance (Omron BF302, Milton Keynes, U.K.); and blood pressure by mercury sphygmomanometer were recorded. An adipose biopsy sample was then washed with diethyl pyrocarbonate and stored at −80°C until analysis.

**Laboratory methods.**

**Biochemical assays.** Mouse glucose and insulin were measured by hexoassay (Thermo Electron, Victoria, Australia) and ELISA (Crystal Chem Inc., Downers Grove, IL), respectively.

Human plasma cortisol was measured using the ImmunoChem cortisol 125I radioimmunoassay coated tube kit (MP Biomedicalals, High Wycombe, U.K.). Enzyme immunoassays (Eurogentec Tashah Corp. UK Ltd., Hampton, U.K.) were used to measure plasma insulin and C-peptide. Electrolytes and salicylate were measured using dry-slide chemistry with a Vitros 950 (Ortho Clinical Diagnostics, Raritan, NJ), and glucose was determined on a Cabas Mira Plus (Roche, Mannheim, Germany). Triglycerides, total cholesterol, and HDL cholesterol were measured in 24-h samples by electron impact gas chromatography/mass spectrometry as previously described (40). The balance between 11β-HSD activities in all tissues was assessed as the ratio of 11β-HSD activity to the mean activity in omental adipose tissue in C57Bl/6 DIO mice, with a similar trend in mesenteric adipose tissue (Fig. 3A).

**Quantification of mRNA.** For cultured cells and mouse tissue samples, total RNA was extracted using TRIzol reagent (Invitrogen, Paisley, U.K.) and the QIAamp RNA Mini Kit (QIAGEN, West Sussex, U.K.). Northern blot analysis was performed using the Qx Gene 5000 (Oxford Clinical Diagnostics, Raritan, NJ), and gel imaging was done using a Chemidoc XRS (Bio-Rad Laboratories, Hemel Hempstead, U.K.). Gene expression was quantified using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18S rRNA. The results are expressed as the ratio of gene expression to the mean expression in omental adipose tissue in C57Bl/6 DIO mice, with a similar trend in mesenteric adipose tissue (Fig. 3A).
pattern of salicylate-induced changes was similar in omental adipose (Fig. 3A). With the exception of increasing AdiQ mRNA levels, these effects were absent in subcutaneous adipose tissue (Fig. 3C).

Compared with salicylate-treated C57Bl/6 DIO mice, HSD1KO-DIO mice had a similar pattern of transcript changes in visceral adipose, with increased AdiQ and reduced TNF-α and ATGL mRNA levels (Fig. 3). However, in HSD1KO-DIO mice, salicylate treatment did not alter mRNA levels of any of the genes affected in C57Bl/6 DIO mice (Fig. 3).

Salsalate downregulates adipose 11β-HSD1 in vivo in men. Characteristics of the 16 male participants are shown in Table 2. Salicylate levels in plasma averaged 134 ± 63 mg/L during active treatment and were undetectable during placebo. Only 1 of the participants reported tinnitus during salsalate therapy, and there were no changes in indices of insulin sensitivity or lipid profile. Nevertheless, salsalate reduced 11β-HSD1 mRNA levels in subcutaneous adipose, an effect which was unrelated to BMI (Fig. 4) or plasma salicylate levels (data not shown). This was accompanied by reduced transcript levels for MCP-1 (33.6 ± 5.6% suppression, \( P < 0.02 \)), but not TNF-α or AdiQ. The effect of salsalate on 11β-HSD1 activity appeared restricted to adipose tissue since there was no change in first pass conversion of cortisone to cortisol in liver or in the ratio of cortisol to cortisone metabolites in urine (Fig. 4).

Salicylate-induced upregulation of AdiQ is mediated by reduced 11β-HSD1 in human adipocytes. In fully differentiated human SGBS adipocytes, 24-h incubation with salicylic acid in the absence of steroid dose dependently reduced 11β-HSD1 mRNA levels (Fig. 5A) but had no effect on AdiQ mRNA (Fig. 5B). Both cortisol and cortisone treatment (0.1 \( \mu \)mol/L for 24 h) reduced mRNA

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TABLE 1

Effects of salicylate on body weight and fasting plasma biochemistry in mice, with biochemical indices, age, high-fat diet duration, and weight measurements for cohorts of mice

<table>
<thead>
<tr>
<th>C57Bl/6 Lean</th>
<th>C57Bl/6 DIO</th>
<th>HSD1KO-DIO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vehicle</strong></td>
<td><strong>Salicylate</strong></td>
<td><strong>Vehicle</strong></td>
</tr>
<tr>
<td>Weight at start (g)</td>
<td>28.8 ± 0.6</td>
<td>29.2 ± 0.5</td>
</tr>
<tr>
<td>Weight at end (g)</td>
<td>29.2 ± 0.8</td>
<td>29.5 ± 0.7</td>
</tr>
<tr>
<td>Age at end (weeks)</td>
<td>17.0 ± 0.0</td>
<td>17.0 ± 0.0</td>
</tr>
<tr>
<td>Time on HFD (weeks)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Fasting plasma</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>0.95 ± 0.11</td>
<td>1.45 ± 0.20*</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>112.5 ± 7.8</td>
<td>113.2 ± 7.1</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.59 ± 0.04</td>
<td>0.63 ± 0.04</td>
</tr>
</tbody>
</table>

Data are mean ± SEM for \( n = 7–8 \) per group. Comparisons were by two-way ANOVA with Bonferroni post hoc tests. HFD, high-fat diet. *\( P < 0.05 \) vs. vehicle of same group. ###\( P < 0.01 \) vs. C57Bl/6 Lean vehicle. ####\( P < 0.001 \) vs. C57Bl/6 Lean vehicle. \( \phi P < 0.05 \) vs. C57Bl/6 DIO vehicle.

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FIG. 1. Effects of salicylate on insulin sensitivity in C57Bl/6 Lean and DIO mice. Plasma glucose (A and C) and insulin (B and D) after intraperitoneal glucose injection in C57Bl/6 Lean, C57Bl/6 DIO, and HSD1KO-DIO mice. Area under the curve (AUC) for all groups shown for glucose (C) and insulin (D). Data are mean ± SEM for \( n = 7–8 \) per group for individual time points. Comparisons for AUC were by two-way ANOVA with Bonferroni post hoc tests. **\( P < 0.01 \) vs. vehicle in same group; #\( P < 0.05 \), ##\( P < 0.01 \) vs. Lean vehicle; \( \phi P < 0.05 \) vs. C57Bl/6 DIO vehicle.
levels of AdiQ. In the presence of cortisone, coincubation with salicylic acid attenuated the suppression of AdiQ, whereas salicylate had no effect on suppression of AdiQ by cortisol (Fig. 5B).

**DISCUSSION**

These results identify a novel mechanism by which salicylates may enhance insulin action in diet-induced obesity, involving downregulation of adipose 11β-HSD1 expression. This downregulation occurs selectively in adipose tissue depots in mice and in humans in vivo, is likely mediated directly within adipose, and is mimicked in human adipocytes in culture. It is crucial that mice deficient in 11β-HSD1 display similar changes in in vivo metabolism and intra-adipose gene expression as salicylate-treated wild-type mice, and salicylate has no additional effects on these parameters in the absence of 11β-HSD1, implicating 11β-HSD1 as a mediator of the beneficial effects of salicylate.

The magnitude and components of the metabolic effects of salicylate remain somewhat contentious. For example, in humans, in addition to reports of improved insulin sensitivity (10,6,41,7), several reports describe worsening of insulin resistance (42–44) or attribute improved metabolism to an increase in insulin concentrations (9). Here, we confirm variable efficacy of salicylate in different settings. In lean mice, salicylate modestly increased plasma insulin concentration but had no other metabolic effects.

In DIO mice, salicylate lowered fasting plasma glucose concentrations and improved glucose tolerance, as well as reducing 11β-HSD1 in visceral adipose (omental and mesenteric) but not subcutaneous adipose tissue. These effects, together with the lack of change in plasma insulin concentrations, are consistent with insulin sensitization primarily in visceral adipose tissue due to reduced local glucocorticoid regeneration by 11β-HSD1 (45,46), with secondary improvements in insulin sensitivity in other organs potentially mediated by altered adipokine secretion. The HSD1KO-DIO mice were <2 weeks younger than the C57Bl/6 DIO mice, so it is highly unlikely that age-dependent effects are responsible for the discrepancies in responses to salicylate between groups. In contrast, in the human study, although we did not undertake sensitive measurements of insulin sensitivity, such as euglycemic clamps, there were no measured effects of salsalate on fasting insulin or lipid profile; this was the case even if we examined only obese patients (not shown). It may be that high doses are required to induce robust insulin sensitization in humans: the salicylate concentrations achieved in this study (mean 134 mg/L, ~1 nmol/L) (Table 2) are lower than in some other studies (e.g., 270 mg/L) (41). This inconsistency is in keeping with a mechanism of action mediated by 11β-HSD1 downregulation, since recent phase 2 clinical trials of selective 11β-HSD1 inhibitors demonstrate that metabolic effects are of relatively small magnitude and not always statistically significant (26,47). Previous studies demonstrating efficacy of salicylates in rodents use genetic models of obesity, including Zucker obese rats and ob/ob mice (32,2,18,3). To our knowledge, this is the first study to demonstrate the insulin-sensitizing

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**FIG. 2. Effects of salicylate on 11β-HSD1 in DIO mice.** 11β-HSD1 mRNA expression in tissues from C57Bl/6 DIO mice (A). 11β-HSD1 enzyme activity in mesenteric and subcutaneous adipose tissue from C57Bl/6 DIO mice (B). Om, omental; Mes, mesenteric; SC, subcutaneous. Data are mean ± SEM for n = 4–8 per group. Comparisons were by Student unpaired t tests. *P < 0.05, **P < 0.01 vs. vehicle in same tissue.

**FIG. 3. Effects of salicylate on adipose mRNA levels in mice.** mRNA levels were measured for AdiQ, TNF-α, MCP-1, lipoprotein lipase (LPL), adipose triglyceride lipase (ATGL), and angiotensinogen (AGT) by qPCR in omental (A), mesenteric (B), and subcutaneous (C) adipose tissue from C57Bl/6 DIO and HSD1KO-DIO mice. Data are mean ± SEM for n = 4–8 per group. Comparisons were by two-way ANOVA with Bonferroni post hoc tests. *P < 0.05, **P < 0.01 vs. vehicle of same genotype; #P < 0.05; #P < 0.01; #P < 0.001 vs. C57Bl/6 DIO vehicle.
INSULIN SENSITIZATION BY SALICYLATE VIA 11β-HSD1

TABLE 2

<table>
<thead>
<tr>
<th>Placebo</th>
<th>Salsalate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>40.1 ± 10.0</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>31.9 ± 8.0</td>
</tr>
<tr>
<td><strong>Waist-to-hip ratio</strong></td>
<td>0.93 ± 0.23</td>
</tr>
<tr>
<td><strong>Percent body fat</strong></td>
<td>25.9 ± 6.5</td>
</tr>
<tr>
<td><strong>Systolic BP (mmHg)</strong></td>
<td>136.8 ± 34.2</td>
</tr>
<tr>
<td><strong>Diastolic BP (mmHg)</strong></td>
<td>79.7 ± 19.9</td>
</tr>
<tr>
<td><strong>Fasting plasma</strong></td>
<td></td>
</tr>
<tr>
<td>Salicylate (mg/L)</td>
<td>ND</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.7 ± 1.2</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.7 ± 0.7</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.2 ± 1.3</td>
</tr>
<tr>
<td>Insulin (mU/L)</td>
<td>26.5 ± 6.6</td>
</tr>
</tbody>
</table>

Data are mean ± SEM for n = 16 per group. Comparisons were by Student paired t tests. BP, blood pressure; ND, not detected. *P < 0.01 vs. placebo.

effects of salicylate in a nongenetic model of obesity. Indeed, diet-induced obesity was chosen because it is a more relevant model to the obesity seen in humans. Furthermore, salicylates improve insulin sensitivity during lipid-induced insulin resistance (2,7). 11β-HSD1 activity is increased acutely in adipose tissue by intralipid infusion (48), potentially contributing to lipid-induced insulin resistance. Blockade by salicylates of the effective increase in 11β-HSD1 activity could therefore explain its relatively selective effect on lipid-induced insulin resistance.

Protective metabolic effects of 11β-HSD1 deficiency in mice receiving a high-fat diet have been described previously (22) and are shown here to bear remarkable similarity to the pattern of effects of salicylates on glucose tolerance and adipose lipase expression. It might be argued that the lack of effect of salicylates in HSD1KO mice is confounded by the improved metabolic “baseline” in these animals. However, to avoid this, we administered high-fat diet for long enough to achieve similar body weight in HSD1KO mice to that observed in C57Bl/6 DIO mice and, thus, ensured these HSD1KO-DIO mice were markedly hyperinsulinemic compared with lean mice. The striking lack of efficacy of salicylate treatment in HSD1KO mice is therefore consistent with downregulation of 11β-HSD1 playing a key role in its mechanism of action.

Downregulation of 11β-HSD1 by salicylates is likely to reflect a direct mechanism in adipose tissue, given its replication in an adipocyte cell line. Furthermore, we have demonstrated in vitro that salicylic acid reverses cortisone-mediated suppression of the insulin-sensitizing adipokine AdiQ, since salicylic acid had no effect when the contribution of 11β-HSD1 was negated by removal of steroid or by addition of cortisol, these data indicate that inhibition of 11β-HSD1 by salicylates mediates the altered AdiQ expression also observed in vivo. The downregulation of 11β-HSD1 was selective for the visceral adipose depots in mice but was evident in subcutaneous adipose in humans. Central adipose depots exhibit greater variation in lipolytic activity and more intense proinflammatory responses during high-fat feeding and are more glucocorticoid responsive (49). However, in humans, recent evidence suggests that 11β-HSD1 regenerates more cortisol in subcutaneous than in visceral adipose tissue (50), suggesting species-specific differences. Moreover, we did not find evidence that changes in proinflammatory cytokines, such as TNF-α, mediate the effects of salicylates on 11β-HSD1 expression; the downregulation of TNF-α by salicylates in C57Bl/6 DIO mice, but resistance to this effect in 11β-HSD1KO mice, suggests that effects of salicylates on intra-adipose inflammation may be a consequence rather than cause of downregulation of 11β-HSD1. This is further supported by the downregulation of TNF-α in the visceral adipose of 11β-HSD1KO mice compared with C57Bl/6 mice. In human adipose, TNF-α mRNA
did not change in association with altered 11β-HSD1 mRNA. In the absence of cytokine-mediated regulation, more detailed investigation will be required to dissect the molecular mechanisms behind the salicylate-mediated regulation of 11β-HSD1 expression.

In conclusion, these findings suggest that the anti-inflammatory agent salsalate alters glucocorticoid metabolism in mice and humans in a pattern that differs between liver and subcutaneous adipose tissue. Downregulation of intra-adipose 11β-HSD1 may contribute to the insulin-sensitizing effect of salicylates.

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M.N. conducted the experiments, analyzed data in mice and in vitro, and wrote the manuscript. D.E.L. and C.L.E. conducted the experiments and analyzed data in mice and in vitro. D.J.W., R.H.S., and R.A. conducted the experiments and analyzed data in humans. J.R.S. and K.E.C. contributed to discussion. B.R.W. wrote the manuscript, which was reviewed by all authors. B.R.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Parts of this study were presented at ENDO 2010 93rd Annual Meeting and Expo of The Endocrine Society, San Diego, California, 19–22 June 2010, and at the British Endocrine Society Meeting, Manchester, U.K., 15–18 March 2010.

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